VOSSIUS & PARTNER

Patentanwälte Rechtsanwälte

Siebertstr. 3 / 81675 München / Deutschland
Tel: +49-(0)89-413 04-0 / Fax: +49-(0)89-413 04-111
E-MAIL: info@vossiusandpartner.com
www.vossiusandpartner.com

PCT/EP2004/012017
Selecore GmbH
Our Ref.: H 2768 PCT S3

English translation of PCT/EP2004/012017 as originally filed

Method for the identification of enzymes with desired characteristics by anchoring the reaction products on the surface of enzyme-presenting organisms

The present invention relates to a method for the identification of enzymes with a desired activity by random production of a large collection of enzyme variants, the synthesis of these variants in host organisms, their presentation on the surface of the organisms and the isolation of enzyme variants with the desired characteristics by detection of the covalent deposition of the reaction product on the surface of the host organism.

The present invention particularly relates to a method for the identification of hydrolases with desired characteristics, wherein the hydrolase variants are presented on the surface of organisms which are decorated with a second enzyme which acts as helper enzyme. The product released by the hydrolase is, in turn, the substrate for the helper enzyme, which activates the product in such a manner that it is covalently fixed by linking to functional groups on the surface of the host organisms. By the fixing of the product, the host organism, which exhibits the desired enzyme activity, is labelled and can then be isolated from a large collection of organisms which express different hydrolase variants. This method is in particular suitable for the screening of large libraries and it allows for the testing of several million enzyme variants in a short period of time and thus, for arriving at the enzyme variants with desired characteristics faster and in a more directed manner than it was previously possible.

Hydrolytic enzymes and in particular esterases and lipases represent a class of enzymes which have become an indispensable adjuvant for a variety of uses in organic chemistry and biotechnology. Their potential is based on their ability to

catalyse not only the hydrolysis but also the synthesis of a lot of different esters which is normally carried out with a high specificity and selectivity. Lipases (EC 3.1.1.3) are carboxyesterases, which have the ability to hydrolyse long-chain acylglyceryl esters (>C_{10}), whereas esterases (EC 3.1.1.1) hydrolyse ester substrates of fatty acids with shorter chains (<C_{10}).

Hydrolases, among them above all lipases and esterases, are used in biotechnology above all for the catalysis of stereoselective conversions of a large number of amine, as well as primary and secondary alcohols (Rogalska et al., Biochem. Soc. Trans. 25 (1997), 161-164; Jaeger et al., FEMS Microbiol. Rev. 15 (1994), 29-63). However, not for every desired transformation reaction is there a corresponding selective hydrolase known. Thus, new paths have been opened up in order to isolate hydrolase mutants from natural sources or to transform known hydrolases in such a manner that they meet the requirements regarding selectivity and specificity for commercial use.

Selection, i.e. cell cultivation under conditions which only allow cell propagation if a certain enzyme is active, has for many years been the method of choice for the isolation of enzymes with desired characteristics. The method which has been most successful until now is based on the mutagenesis of a gene *in vitro* which codes for a desired enzyme, on the introduction of the mutagenised DNA into cells in order to generate a library and finally on the selection of cells which produce an active enzyme by means of applying restrictive growth conditions. Thus, e.g. Palzkill et al. (J. Bacteriol. 176 (1994), 563-568) constructed collections of large libraries in which sequence sections comprising several amino acids have been randomised in beta lactamase by the use of *in vitro* techniques. The beta lactamase mutant genes were introduced into *E. coli* by transformation. Cells, which were able to grow in the presence of beta lactam antibiotics which are normally bad substrates for beta lactamase, were isolated (Venkatachalam et al., J. Biol. Chem. 269 (1994), 23444-23450).

A large number of techniques, including chemical mutagenesis of isolated DNA, gene amplification by error-prone PCR and oligonucleotide mutagenesis were used in order to generate libraries of mutant genes which exhibited a desired number of nucleotide substitutions. Often, several rounds of selection and mutagenesis are used in order to select enzymes which are more and more improved.

The selection of improved enzymes which originate from gene libraries which have been constructed in vitro is a potent method to obtain enzymes with desired characteristics. However, this method can only be used if the desired enzyme

catalyses an essential reaction which is also vital for the surviving of the cell. Unfortunately, in the case of a large number of enzyme-catalysed reactions which are interesting from a commercial point of view, it is not possible to develop a selection strategy.

For enzymatic reactions, where the design of a selection strategy is not possible, mutant libraries have to be screened using a direct assay. Here, in turn, a collection of enzymatic genes is created by the introduction of mutations into the gene of the corresponding enzyme. By individually introducing these genes, normally in the context of an expression plasmid, into a microbial expression host, a population of microorganisms is generated in which each clone, in principal, synthesises an enzymatic variant varying with respect to the amino acid sequence. The individual clones are then propagated separately, either as colonies on agar plates or in 96-well plates. The host cells are lysed and, thus, the enzymatic variant is released. Each individual lysate is then incubated with a given – often chromogenic – substrate molecule and the transformation reaction is measured. Such microorganisms producing an enzyme variant which exhibits desired characteristics with respect to the desired enzymatic characteristics are, subsequently, propagated. By obtaining the genetic sequence coding for the enzyme variant and by determining the base sequence of the gene which has been modified as compared to the wildtype, the variant obtained can be characterised regarding its deviation from the base sequence of the gene which had been originally used. By using this strategy, e.g. Moore and Arnold (Nature Biotechnol. 14 (1996), 458-467) could isolate a variant of the p-nitrobenzylesterase, which in 30% DMF exhibited a 16-fold higher activity than the starting enzyme.

Usually, the enzyme variant obtained from a single round of mutagenesis and screening shows slight improvements of its enzymatic characteristics in the direction of the desired, however, the desired characteristic is not fully developed. Thus, mostly, on the basis of the enzyme variant obtained, a new set of gene variants is generated and these are again subjected to the process of gene expression in microorganisms, determination of the enzymatic characteristics of individual clones and identification of variants which are improved with respect to the desired characteristics, until the desired characteristic is obtained.

However, this method has a substantial disadvantage: It requires a physical separation and cultivation in physically separated culturing containers (e.g. microtiter plates or test tubes) of the individual microbial clones which carry randomly modified genes for the enzymes and which, thus, also produce different

enzyme variants. In each of the separated culturing containers, the enzymatic characteristic of the enzyme variant produced by the corresponding microbial clone has to be measured. Due to the separate handling of the microbial clones and the separated determination of the enzymatic characteristics of each microbial clone, which is consequently necessary, this method is very time and money consuming. Thus, for reasons of logistics and costs, only one subset of the actually produced gene variants can be screened. Usually, several hundred to several thousand clones are screened as described above with respect to the desired characteristic. The variety of actually produced gene variants is in comparison therewith considerably higher and can amount to above 10^9. However, it is very desirable to be able to screen as many enzyme variants as possible as otherwise, there is the risk that one variant with especially suitable characteristics remains undiscovered because it does not pertain to the group of the screened enzyme variants.

Apart from the fact that only a limited number of screening reactions can be carried out, the screening of colonies using plate assays has also other limitations: as overwhelming number of proteins are not released by *E. coli* - the most suitable host organism for a directed evolution – the substrate has to be able to diffuse into the cells and it must not be toxic. Moreover, plate assays, also those for which fluorescent molecules are used, often exhibit a moderate specificity.

Thus, there is the tendency to develop ultra-high throughput methods which (1) work on a small reaction scale, (2) allow a quick measurement of the enzymatic characteristics of each individual clone of a microbial population and with which (3) a far larger amount of clones can be treated than according to the state of the art. In this respect, it is desirable that the enzyme variant to be tested is not only synthesized but also released by the microbial producer, so that a direct interaction with the substrate can take place and moreover, almost any reaction condition with respect to the selection of the solvent, pH, ion concentration, etc. can be created.

Another aim is, moreover, to provide the enzyme to be examined outside of the producing cell in a form that it is covalently linked to the surface of the producing cell. During the past years, a number of screening methods for the isolation of enzymes with improved characteristics have been developed which have the aim of removing the limitations of plate assays and which are based on the screening of surface-exposed enzyme libraries. The use, which is possibly the most innovative, has been described using the protease OmpT from *Escherichia coli* (Olsen *et al.*, Nat. Biotechnol. 18 (2000), 1071-1074) as an example. Here, with OmpT, a protease from *Escherichia coli* was used which is per se a protein of the outer membrane and is, thus, bound to the outer membrane of *E. coli* and exposes a

protease domain at the outside of the bacterial surface. A random collection of *ompT* gene variants was produced. The *ompT* variants operatively linked thereto were expressed and the corresponding enzymatic protein domain variant on the surface of the corresponding bacterial cell producing it, is exposed. The bacterial cells were incubated with a synthetic peptide carrying two physically adjacent fluorophores. By hydrolytic cleavage – caused by an OmpT variant with the desired substrate specificity – of the peptide, a separation of the fluorophores takes place and thus, a change in the fluorescence properties of the product compared to the substrate which then exhibits an increased green fluorescence. The released product has a positive net charge so that it remains bound to the (negatively charged) surface of the cell which exhibits a proteolysis activity vis-à-vis the substrate. Thus, it was possible to isolate cells which show green fluorescence by means of flow cytometry, in the case of which the observed green fluorescence correlates with a catalytic activity of the surface-exposed OmpT protease variant. A substantial disadvantage of this method is, however, that the reaction product released by the reaction with the enzyme is only bound by ionogenic interactivities to the surface of the bacterial cell converting the substrate. Thus, a desired permanent coupling of the phenotype of the enzymatic activity with the corresponding genotype of the bacterial cell expressing this activity is not given. There is always the risk that product molecules detach themselves from the surface of the bacterial cell which generates them, are, thus, released in solution and, thus, bind to the surface of other bacteria which do not express the desired enzymatic activity. Thus, an identification of the bacteria expressing the desired enzymatic activity is rendered considerably more difficult by the detection of the bound product molecules. Moreover, the binding of the product to the cell surface only remains intact under low salt conditions and the method is only applicable if the product obtained has a charge and if this charge is opposite to the charge of the surface of the host cell. Moreover, it is only applicable to such microorganisms which have a charged surface. Furthermore, the method cannot be used if the charge on the substrate or product molecule has a disadvantageous effect on the enzymatic reaction.

Thus, there is the need to provide methods for the isolation of enzymes, in particular of enzymes with substrate-cleaving activity, with desired characteristics which allow an extremely high throughput and a simple and reliable determination of positive clones.

The problem underlying the present invention is, thus, to provide such methods.

This problem is solved by providing the embodiments indicated in the claims.

Thus, the present invention relates to a method for the identification of an enzyme with a desired substrate-cleaving activity, wherein a library encoding a plurality of different polypeptide candidates is expressed by suitable host organisms in such a manner that the polypeptide candidates are presented on the surface of the host organisms and the host organisms are contacted with the substrate to be cleaved, characterised in that

(a) on the surface of the host organisms a helper enzyme is provided which allows the formation of a covalent bond between the surface of the host organism and a product created by the substrate cleaving reaction which is catalysed by a polypeptide candidate and

(b) the identification of the host organisms which have the product bound to their surface.

The method according to the invention is particularly advantageous as it allows the covalent bond of a product of the reaction catalysed by the polypeptide candidate to the surface of the host organism. Thus, the reliability with which host organisms expressing the desired enzymatic activity can be identified, is considerably increased. The covalent fixing of the reaction product to the surface of the host organism allows a better correlation between the detection of the desired enzymatic activity and the host organism expressing this enzymatic activity. The disadvantages of the methods described in the prior art, in which the reaction product is bound by ionic interactions to the cell surface of a host cell, are avoided. The method of the invention is, moreover, widely applicable, i.e. to all possible enzymatic activities and substrates as the charge of the substrate or of the host organism used does not have to be taken into consideration.

In the context of the present invention, the term "enzyme" means a polypeptide which is able to catalyse a biochemical reaction.
The method according to the present invention can be used for the identification of enzymes with substrate-cleaving activity. In this context, the term "substrate-cleaving" means that the enzyme catalyses a reaction by which a given substrate (educt) is cleaved into at least two products. Reactions, leading to the cleavage of an educt by an enzyme into at least two products are, for example, hydrolysis, phosphorolysis or elimination. Here, phosphorolysis is the cleavage of a bond by

orthophosphate. Hydrolysis relates to a reaction where the cleavage of a bond is carried out by water, wherein an OH group is incorporated into a product of the cleavage reaction and a hydrogen atom into the other product. Elimination relates to a reaction in which two substituents of a pair of adjacent atoms in a molecule are removed without substitution by other atoms or groups.

In a preferred embodiment of the method of the invention, the enzyme with substrate-cleaving activity therefore has hydrolase activity, i.e. it is able to catalyse a hydrolysis reaction. Examples of enzymes with hydrolase activity are esterases, lipases, phosphatases, glucosidases, acylases or amidases. Phosphatases are hydrolases by which phosphoric acid monoesters are hydrolytically cleaved from e.g. sugar phosphates, nucleoside monophosphates or as terminal phosphate group from nucleic acids. Depending on the pH at which the effect of these enzymes is best, a differentiation is made between acid phosphatases and alkaline phosphatases. Glucosidases are hydrolases which cleave glycosidically bound glucose. Amidases catalyse the hydrolytic cleavage of amides. Acylases are enzymes which are able to catalyse the cleavage of acyl groups from a molecule. E.g. deacylases but also lipases are members of this group.
Lipases (E.C. 3.1.1.3) are carboxylic esterases with the ability to hydrolyse long-chain fatty acid esters ($\geq C_{10}$). Esterases (E.C. 3.1.1.1), on the other hand, have the ability to hydrolyse ester substrates of shorter-chain fatty acids ($\leq C_{10}$). Among these are e.g. lipases and esterases which are important in the field of biotechnology such as phospholipases (leather processing), acylases from B. megaterium and E. coli (chemical synthesis), lipase from Aspergillus sp. (prostaglandin synthesis), LipA from B. subtilis (cephalosporine synthesis), lipase from Candida sp. (pyrolidinion synthesis), lipase from C. rugosa (synthesis of ibuprofen), from chromobacterium (vitamin D synthesis), from M. miehei (synthesis of ketoprofen), from P. cepacia (rapamycin synthesis), from P. fluorescens (synthesis of hydantoins) and from streptomyces sp. (synthesis of penicillins).

Preferably, the enzyme with esterase activity is derived from an esterase from a procaryotic organsim, preferably from a bacterium, particularly preferred from a bacterium from the genus Pseudomonas and more particularly preferred from the species Pseudomonas aeruginosa. Particularly preferred, the esterase is derived from esterase EstA from Pseudomonas aeruginosa. This enzyme is e.g. described in Wilhelm et al. (J. Bacteriol. 181 (1999), 6977-6986). The nucleotide and amino acid sequence of EstA esterase are shown in figure 4.

In another preferred embodiment, the enzyme with lipase activity is derived from the lipase LipA from Bacillus subtilis (Eggert et al., Eur. J. Biochem. 267 (2000), 6459-6469; Van Pouderoyen et al., J. Mol. Biol. 309 (2001), 215-216; Eggert et al., FEBS Lett. 502 (2001), 89-92; Eggert et al., FEMS Microbiol. Lett. 225 (2003), 319-324).

The substrate used in the method of the invention and for which an enzyme with the corresponding substrate-cleaving activity is desired, can, in principle, be any substrate which is cleavable by an enzyme. In the method of the invention, the substrate is selected in such a manner that it is cleaved by the desired enzymatic activity to be identified. Moreover, in this context, it should be verified that the substrate only serves as substrate for the desired enzymatic activity to be identified, not, however, for the helper enzyme which is at the same time provided on the surface of the host organism. Preferably, it is a substrate which is hydrolytically cleavable by a reaction catalysed by an enzyme, particularly preferred the substrate is an ester. If during the method it is tried to identify an enzyme which is an esterase or a lipase, the substrate if preferably an ester of shorter-chain fatty acids ($\leq C_{10}$) or a derivative of such an ester or of a long-chain fatty acid ($\geq C_{10}$) or a derivative of such an ester. Examples of derivatives are e.g. halogenated fatty acids, branched-chain fatty acids, amino acids or hydroxy acids and many others.
In a preferred embodiment of the method of the invention, the enzyme has esterase or lipase activity and the substrate is a phenol derivative of an ester of any carboxylic acids, i.e. a phenol ester.
Preferably, the substrate has a constituent which, after cleavage by the desired enzymatic activity, leads to a product which can be transformed by the helper enzyme into an activated form, which, then, covalently binds to groups on the surface of the host organism. Such constituents are known to the skilled person, e.g. from the US patent 5,196,306 and e.g. comprise tyramine and p-hydroxyphenylpropionyl biocytin. In the case of an ester, e.g. the alcohol component can be transformed by a helper enzyme into a radical after cleavage by an esterase.
In a particularly preferred embodiment of the method of the invention, the substrate to be used carries a constituent allowing the detection of the product of the substrate cleavage reaction. Examples of such a constituent are markers, which are routinely used in molecular biology and which allow a detection, e.g. fluorescence marker, chemiluminescence marker, radioactive marker, biotin, avidin, streptavidin,

antigenes for antibodies, magnetic particles or an enzyme which leads to a detectable dye upon contact with a chromogenic substance. Such markers and their uses are known to the skilled person and are also described in connection with the identification of new desired enzymatic activities in the US patent application 20030036092. The constituent which allows the detection of a product of the substrate cleavage reaction is, in this context, situated at the substrate on a component which, after the cleavage reaction, is covalently fixed to the surface of the host organism by the helper enzyme. If, e.g. a phenolic ester is used as substrate and a peroxidase as helper enzyme, the alcohol function in the phenolic ester can be linked to a detectable signal molecule (in the example indicated biotin). The tyramide which is set free by the hydrolysis of the ester and which carries the signal molecule is activated in the presence of H_2O_2 by the peroxidase which is fixed to the surface of the host organism. The phenol radical reacts with aromatic residues on the surface of the host organism and is thereby covalently fixed. The presence of the product, marked with the signal molecule, on the surface of the host organism can then be detected with detection methods known to the skilled person.
In the case of biotin, the detection can, e.g. be carried out by the formation of biotin/streptavidin conjugates. Streptavidin can e.g. be coupled to a fluorescent substance, e.g. R-phycoerythrin, which allows the detection of biotin by the formation of biotin/streptavidin conjugates. Cells which have the corresponding fluorescence-marked conjugates on their surface can be identified by flow cytometry, e.g. fluorescence-activated cell sorting, and isolated.

Alternatively, also the substrate itself can be linked to a fluorescence dye so that the product fixed to the cell surface by the helper enzyme is fluorescence labelled. In this case, too, the fluorescence labelled cells can be identified by flow cytometry, e.g. fluorescence-activated cell sorting, and isolated.

According to the invention, a "helper enzyme" is provided on the surface of the host organism presenting a candidate molecule on its surface. This helper enzyme is able to catalyse a reaction which allows the covalent bond of a product of the cleavage reaction on the surface of the host organism. Preferably, the helper enzyme is an enzyme which is able to transform a product set free by a substrate cleavage reaction into an activated from, which is able to covalently bind to groups which are present on the surface of the host organism. Such enzymes are known to the skilled person and preferably comprise those described in US patent 5,196,306,

the disclosure content of which is hereby incorporated by reference into the present application. Examples of helper enzymes are, thus, peroxidases, ligases, oxidoreductases, transferases and isomerases. Peroxidases, oxidases, e.g. aminooxidases and transferases are preferred. The helper enzyme is preferably a peroxidase. The term peroxidase generally refers to enzymes which catalyse the oxidation of a compound with peroxide as oxidation agent.
In principle, any given peroxidase can be used in the method of the invention. Examples are myeoloperoxidase (McCormick et al., J. Biol. Chem. 273 (1998), 32030-32037; myeoloperoxidase from human leucocytes is marketed by Fluka, Sigma-Aldrich), lactoperoxidase (Heinecke, Toxicology 177 (2002), 11-22; Ostdal et al., J. Agric. Food Chem. 48 (2000), 3939-3944; lactoperoxidase from cow milk is marketed by Fluka, Sigma-Aldrich), ribonuclease A if nickel ions are added (Gill et al., Chem. Res. Toxicol. 10 (1997), 302-309) and peroxidase from horseradish (Horeseradish-Peroxidase; HRP).
Horseradish peroxidase (HRP) is preferably used. It can be obtained cheaply (e.g. Sigma, Fluka) and proved successful for a large number of biochemical detection reactions.

As mentioned above, the helper enzyme is preferably able to transform a product set free by the substrate cleavage reaction into an activated form which is able to covalently bind to groups which are located on the surface of the host organism. Such groups on the host organism particularly comprise aromatic residues such as the side chains of tyrosine, tryptophan or histidine residues. Here, an "activated form" is preferably understood to mean a highly reactive short-lived reaction product. An activated form can, e.g., be a radical. Radicals have the advantage that they are very quickly deactivated by water molecules if they do not immediately react with molecules on the surface of the host organism. Other activated forms are described in US patent 5,196,306. It is also conceivable to use an aminooxidase as helper enzyme if the product set free by the cleavage reaction carries a free amino group. The aminooxidase subsequently transforms the free amino group into an aldehyde which then reacts on the surface of the organism with primary amines by the formation of Schiff's bases and which can form a covalent bond.
Analogously, other oxidases can also be used as helper enzymes which transform a product set free in a cleavage reaction into an aldehyde, e.g. galactoseoxidase, which transforms galactose which was set free into the corresponding aldehyde.

The term "provided on the surface of the host organism" means that the helper enzyme is present on the surface of the host organism. The helper enzyme can be provided on the surface, according to known methods. For example, an irreversible or a reversible immobilisation of the helper enzyme on the surface of the host organism is possible. An irreversible immobilisation can e.g. be achieved by a covalent binding of the helper enzyme to groups which are present on the surface of the host organism. Thus, it is possible to immobilise a helper enzyme, particularly peroxidase, on the surface of the host organism by carrying out an oxidation of the sugar side chains of the protein with sodium periodate and Schiff's bases reaction of the thus produced sugar aldehydes with primary amino groups which are present on the surface of the host organism. Such a method is e.g. described in Hermanson (Bioconjugate Techniques (1996); Academic Press, New York). Other possibilities of producing a covalent bond are known to the skilled person, e.g. by using glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccininimide ester, carbodiimide or bis-diazotated benzidine. These and other methods for producing covalent bonds are described in "Cross-linking techniques" (Baumert and Fasold, Methods Enzymol. 172 (1989), 584-609).
The formation of the helper enzyme on the surface of the host organism in the form of a conjugate of helper enzyme and receptor is also possible wherein the receptor is able to bind a molecule which is present on the surface of the host organism. An example of such a receptor is an antibody which recognises a structure, e.g. a protein on the surface of the host organism. This can e.g. be a conjugate of the helper enzyme and an anti-E. coli antibody which recognises lipopolysaccharides on the cell surface. Such conjugates are commercially available, e.g. from Maine Biotechnology Services Inc.. Another possibility is a conjugate from a sugar-binding lectin and a helper enzyme (cf. e.g. Appukuttan et al., Biochem. Biophys. 37 (2000), 77-80). Another possibility to provide the helper enzyme on the surface of the host organism is that it is expressed by the host organism in such a manner that it is presented on the surface of the host organism. Methods by which this can be achieved are known to the skilled person and are described in detail below in connection with the expression of the candidate polypeptides.

In a particularly preferred embodiment of the method of the invention, the enzyme to be identified is esterase, the helper enzyme is a peroxidase and the substrate is a phenol derivative of an ester of any given carboxylic acids, i.e. a phenol ester. Such phenol esters have a functional group which allows the detection of the deposition of the product of the esterase activity on the surface of the host

organism. Here, the known fact is used that derivatives of phenol are activated by peroxidase in the presence of H_2O_2 by the formation of a phenolic radical, which form covalent adducts with groups of other molecules which are rich in electrons, such as e.g. tyrosine or tryptophan residues. This characteristic was, for example, used to stain preparations immunohistochemically (Van Gijlswijk et al., J. Immunol. Methods. 189 (1996) 117-127; Bobrow et al., J. Immunol. Methods 125 (1989), 279-285; US patents 6,593,100, 5,731,158 and 5,196,306; EP 1129214). Here, peroxidase is conjugated to a receptor (e.g. an antibody) and is, thus, selectively placed by the receptor binding. Conjugate which was not bound is washed away. Then, a phenolic substrate molecule is added to which a signal molecule, e.g. biotin, is bound. Due to the short lifetime of substrate which was activated by peroxidase, the substrate reacts in proximity of the place of its formation and covalently fixes the biotin at functional groups which are immediately adjacent (Figure 2). The phenol ester itself is not a substrate for the peroxidase. Thus, it is not possible to carry out an activation of the phenol ester by the peroxidase and deposition on the surface of the host organism. Only if, by means of the enzymatic activity presented on the surface of the host organism, the acid function is cleaved and the free phenolic component is present, a covalent binding of the phenolic component, i.e. of the reaction product of the enzymatic activity, on the surface of the host organism is possible.

As has already been described above, the substrate (in particular the part which, after the cleavage reaction, is fixed by the helper enzyme as a product to the surface of the host organism) can be provided with a marker molecule which allows the detection of the product on the surface. Thus, the phenol ester can e.g. be coupled to biotin in such a manner that biotin is fixed to the phenol residue. The biotin-coupled phenol component which is fixed to the surface of the host organism can then be detected with detection methods for biotin known to the skilled person. It is also possible to couple a fluorescence dye to the phenol residue of the phenol ester which, then, allows the direct detection or the isolation of host organisms to the surface of which the fluorescence labelled phenyl component is fixed. In the case of cells, the flow cytometry, e.g. fluorescence-activated cell sorting is possible. If phages are used as host organisms, their enrichment and isolation can be achieved, e.g. by the adsorption to surfaces coated with a corresponding receptor molecule. In this context, synthetic surfaces (e.g. microtiter plates) or magnetic particles are for example possible surfaces. A common method is the detection

using biotin or digoxigenin, whereby the strong binding of biotin to streptavidin or of digoxigenin to an digoxigenin antibody is made use of.

If a peroxidase is used as a helper enzyme in the method of the invention, the medium, in which the host organisms are present at the time of the contacting with the substrate, contains H_2O_2 which is necessary for the enzymatic reaction of the peroxidase. The concentration of H_2O_2 is adjusted in such a manner that it is not toxic for the host organisms. Suitable concentrations are preferably in the range of 0.00005% (v/v) to 0.005% (v/v), preferably in the range of 0.000075% (v/v) to 0.004% (v/v), particularly preferred in the range of 0.00009% (v/v) to 0.003% (v/v), particularly preferred the concentration is in the range of 0.0001% (v/v) to 0.001% (v/v).

So far, methods described for H_2O_2 mediated covalent deposition of phenolic components have been used only for the immunohistochemical staining of fixed cell material, however, not with living cells or other organisms, since H_2O_2 is a strong cell poison and it was to be expected that the H_2O_2 concentration used for a peroxidase reaction was incompatible with the survival of the cells. Surprisingly, it was found that the concentration of H_2O_2 can be adjusted to an extent such that on the one hand, it does not lead to cell death, however, on the other hand allows for an efficient deposition of the phenolic alcohol on the cell surface. It was shown that this method can successfully be used for selective labelling of living cells with the desired esterase activity by means of simultaneous cell surface exposition of enzyme (esterase) and helper enzyme (peroxidase) and the use of phenol esters as substrates, and for isolating cells with the desired enzyme activity from a population of cells without enzyme activity by means of iterative rounds of isolating of labelled cells, growing of isolated labelled cells and re-labelling and isolating.

The host organisms used in the methods according to the invention can be any kind of host organism that is suitable for the presentation of the polypeptide candidates on their surfaces. In principle, any host organism can be used which is able to carry genetic information, to express it and to replicate itself. Thus, the term "organism" comprises any type of cells but also viruses and phages. In a preferred embodiment, the host organisms are cells or phages.
If the host organisms are cells, they can be eucaryotic or procaryotic cells. Preferably, the host organisms are procaryotic organisms, particularly preferred bacteria. In this case, gram-negative bacteria are preferred and amongst these host

organisms of the species *E. coli* are particularly preferred.

In the method according to the invention, host organisms are used which express the polypeptide candidates in such a way that they are presented by the host organisms on their surface. The person skilled in the art knows a plurality of methods for achieving a surface exposure of a protein on a host organism. Such methods are known e.g. under the terms "phage display" and "microbial display". Mostly, the exposure on the surface is based on the fact that by use of suitable genetic engineering methods, an enzyme variant is provided on the surface of the host organism which is covalently bound to a component of the surface. When bacteria are used as host organisms, this is achieved e.g. by the fact that the gene coding for the enzyme which is to be optimised with regard to its properties is operatively linked with the coding sequence for a protein of the outer membrane of a microbial producer, so that a fusion protein is built which is anchored in the outer membrane of the bacterium and exposes the linked protein domain on the outside of the outer membrane. As membrane anchorage domain, amongst others, a fragment of the *E. coli* OmpA protein (Francisco *et al.*, Proc. Natl. Acad. Sci. USA 90 (1993), 10444-10448), an *Escherichia coli* adhesin (Maurer *et al.*, J. Bacteriol. 179 (1997), 794-804) or the intimin from enteropathogenic E. coli (Wentzel *et al.*, J. Bacteriol. 183 (2001), 7273-7284) was used. Methods for the presentation of proteins on the surface of *E. coli* by the presentation as fusion proteins with porins of the outer membrane of *E. coli* were described in particular in Lang (Int. J. Med. Microbiol. 290 (2000), 579-585), Francisco et al. (Proc. Natl. Acad. Sci. USA 89 (1992), 2713-2717) and Wentzel et al. (J. Biol. Chem. 274 (1999), 21037-21043). The presentation as fusion protein with a cellular appendix (fimbrium) was described e.g. in Westerlund-Wikstrom et al. (Protein Eng. 10 (1997), 1319-1326), Westerlund-Wikstrom (Int. J. Med. Microbiol. 290 (2000), 223-230), Kjaergaard et al. (Appl. Envrion. Microbiol. 67 (2001), 5467-5473) and Schembri et al. (FEMS Microbiol. Lett. 170 (1999), 363-371). The presentation as fusion protein with an autotransport protein of the outer membrane is described e.g. in Klauser et al. (EMBO J. 11 (1992), 2327-2335) and Maurer (J. Bacteriol. 179 (1997), 794-804).
Methods for the presentation of polypeptides on the surface of other microorganisms than *E. coli* have also been described. In this context, e.g. Jung et al. (Nat. Biotechnol. 16 (1998), 576-580) and Kim et al. (Appl. Envrion. Microbiol. 66 (2000), 788-793) describe the cell surface presentation of polypeptides on the surface of Pseudonomas syringae by fusion to the ice nucleation protein. Also, methods for the surface presentation of polypeptides in Bacillus subtilis (Hansson

et al., Comb. Chem. High Throughput Screen. 4 (2001), 171-184) and in Staphylococci (Lehtio et al., FEMS Microbiol. Lett. 195 (2001), 197-204) have been described. Also methods for the surface presentation of polypeptides on eucaryotic cells are already known, e.g. for Saccharomyces cerevisiae (Boder and Wittrup, Nat. Biotechnol. 15 (1997), 553-557; Boder and Wittrup, Methods Enzymol. 328 (2000), 430-444).
Preferred methods for the expression of polypeptide candidates leading to the presentation of the polypeptides on the surface of cells are known to the person skilled in the art particularly from the US patent application 20030036092 and from Olsen et al. (Nature Biotechnology 18 (2000), 1071-1074).

The presentation of polypeptides on the surface of phages, the so-called phage display, has been described in detail and is used in many cases (see e.g. Miyakubo et al. (Nucleic Acids Symp. Ser. 44 (2000), 165-166), Widersten et al. (Meth. Enzymol. 328 (2000), 389-404), Widersten and Mannervik (J. Bol. Biol. 250 (1995), 115-122), Korn et al. (Bio. Chem. 381 (2000), 179-181) and Droge et al. (J. Biotechnol. 101 (2003), 19-28)).

The method according to the invention serves the identification of enzymes with a desired substrate-cleaving activity. It creates methods which are known in the state of the art, in which enzymes with desired activities are identified in that in host organisms, a great number of different polypeptides candidates are expressed and the host organisms expressing the desired enzyme activity are determined. As a rule, the production of such host organisms is carried out by the provision of DNA libraries coding for a plurality of polypeptides and the introduction into corresponding host organisms. The DNA libraries can e.g. be produced by the in-vitro mutagenesis of an initial gene coding for a specific enzyme. Due to the mutagenesis, variants of the enzyme are produced which can then, after expression in the host organisms, be tested and selected with regard to their enzymatic properties. Suitable methods for the in-vitro mutagenesis and for the production of suitable host organisms are known to the person skilled in the art and are described in detail e.g. in the US patent application US 20030036092. They comprise e.g. the chemical mutagenesis, in particular of isolated DNA, gene amplification by "error prone" PCR and oligonucleotide mutagenesis. Further possibilities are the ligation of randomised gene segments (cassette mutagenesis), gene shuffling, in vivo mutagenesis with mutagenic agents and the use of E. coli mutator strains. According to the invention, thus, a library is expressed in the host

organisms, whereby this library encodes a plurality of polypeptide candidates.

The identification of the host organisms in step (b) of the method can be carried out according to methods known to the person skilled in the art, as described e.g. in the US patent application 20030036092. If the product produced by the substrate cleavage reaction is in itself a product which can be detected directly or indirectly, then the use of a marker molecule is not absolutely necessary. As discussed above, in the method according to the invention, however, a substrate is preferably used which is linked to a marker molecule which allows the detection of the product which is bound on the surface of the host organism. Examples of such markers are mentioned above and comprise biotin or fluorescent dyes.

Apart from the identification of the host organisms that carry the product, markers of that kind can also allow the isolation of these organisms. Preferably, the isolation is carried out as described in the US patent application 20030036092. In the case of fluorescent dyes which are directly bound to the product, cells carrying a product marked in that manner on their surface can be isolated by flow cytometry, e.g. fluorescence-activated cell sorting. The same method can be applied if the marker coupled to the product is conjugated with another substance which is fluorescent itself or is coupled with a fluorescence marker. An example is the possibility to have biotin as a marker on the product and to detect biotin by streptavidin to which a fluorescence marker is coupled. If phages are used, e.g. an isolation is possible via surfaces carrying an receptor for the marker molecule used, as already described above.

Another possibility for the isolation of the host organisms which have bound (marked) product on their surface is magnetic (cell) sorting. For this purpose, the host organisms are contacted with magnetic particles which, on their surface, carry a molecule which binds to the product fixed on the surface of the host organism or to the marker coupled to the product. Thus, such magnetic particles can e.g., on their surface, carry a biotin-binding molecule (e.g. streptavidin) and would then bind to organisms carrying a product coupled with biotin on their surface.

There is the possibility of propagating the isolated cells/organisms after a first (cell) sorting, contacting them again with substrate and subjecting them again to (cell) sorting. These procedures can be repeated several times in order to obtain an enrichment of the desired cells/organisms.

In step (b), the method according to the invention leads to the identification of host organisms having the product of the substrate-cleaving activity fixed on the surface and, therefore, are expected to express the desired enzyme activity. Starting from

organisms identified in that manner, the method according to the invention can be carried out repeatedly.
For this purpose, the DNA sequence contained in the identified host organisms, which encodes the enzyme activity is used as starting point for the production of a new library coding for a plurality of polypeptide candidates. This can take place by means of mutagenesis methods known to the person skilled in the art, as mentioned above already. Host organisms expressing said library are then again used in the method according to the invention.

The present invention also relates to host organisms which express a polypeptide candidate (enzyme) in such a manner that it is presented on the surface of the host organism and which, at the same time carry a helper enzyme on their surface, which is able to catalyse a reaction which allows the formation of a covalent bond between the surface of the host organism and a product of a substrate cleaving reaction which is catalysed by the polypeptide candidate. For the preferred embodiments of the host organisms, polypeptide candidates (enzymes), helper enzymes and so on, the same applies which has been said above in connection with the method according to the invention.

The disclosure content of the documents mentioned in connection with the description of the invention is incorporated into the present application by reference.

The following examples serve the explanation and the illustration of the invention. The invention is, however, not limited to the embodiments shown in the examples but refers to all the possible embodiments discussed above.

Figure 1: Schematic illustration of the method according to the invention by means of the example of the isolation of cells with esterase activity by covalent deposition of the hydrolysis product on the cell surface. Clockwise: Use of a library of *E. coli* cells carrying, randomly varied esterase genes. After induction of the gene expression, the esterase is presented on the cell surface. Then, the helper enzyme peroxidase is fixed on the surface of the bacteria. Enzyme which has not bound is removed by centrifugation of the bacteria and discarding of the supernatant. Then, an ester substrate is added which is a phenolic ester of which the alcohol function is linked to a detectable signal molecule (here biotin). The biotin tyramide released by hydrolysis of the ester (in Figure 1 illustrated

schematically by a triangle) is activated by the cell-surface-fixed peroxidase in the presence of H_2O_2. The phenol radical reacts with aromatic residues on the cell surface and is thus fixed covalently. Substrate molecules which have not reacted are removed by centrifugation and washing of the cells. The signal molecule fixed on the surface can be detected directly, if it is a fluorescent dye, or detected indirectly, as shown here in the example of the deposition of biotin and its detection by streptavidin, R-phycoerythrin conjugate. Thus, a coupling of the esterase activity of the cell with a detectable cell surface signal takes place, which allows for the isolation of an esterase-active cell from a population of cells, e.g. by use of flow cytometry or magnetic cell sorting.

Figure 2: TSA reaction (Tyramide Signal Amplification). Peroxidase-mediated covalent coupling of biotin-tyramide with a tyrosyl residue of a protein. A biotin molecule linked to a phenol derivative is activated by peroxidase in the presence of H_2O_2 by the formation of a phenyl radical. The radical reacts, as it is very short-lived, with aromatic residues in the proximity of the place of its formation. Thus, a covalent fixation of the detectable signal – here biotin – is achieved.

Figure 3: Structure of LC-LC-biotin-tyramide octanoic acid ester. This ester can be used as substrate molecule for an esterase. Then, the released biotin-tyramide can be activated by peroxidase in the presence of H_2O_2.

Figure 4: Nucleotide sequence and derived amino acid sequence of the esterase gene *estA* from *Pseudomonas aeruginosa* (from Wilhelm et al., *J. Bacteriol.* 181 (1999), 6977-6986). The coding amino acid sequence begins with base 206. The putative signal sequence is marked by an arrow.

Figure 5: Cell surface presentation of EstA. For the detection of the cell surface exposure of EstA, induced *E. coli* cells expressing the *estA* gene under *lac* promoter control were incubated with anti-EstA antibodies and stained with a biotinylated second antibody and streptavidin, phycoerythrin conjugate. The immunofluorescence of the cells was analysed in a Zeiss Axioscope (filter 15). Left: fluorescence; right: transmitted light picture.

Figure 6: Esterase-mediated deposition of biotin on the surface of *E. coli*

cells. Induced *E. coli* cells carrying both peroxidase and EstA on their surface (*E. coli* pBBX+) and equally treated control cells not containing an estA gene (pBBR1MCS) were incubated with the substrate octanoic acid-biotin-LC-LC-tyramide ester (Figure 3) in the presence of 0.001% H_2O_2. Cells of the same *E. coli* strain carrying no estA gene were used as controls. The cells were washed in PBS buffer after 15 min incubation and stained with streptavidin, phycoerythrin conjugate. The immunofluorescence of the cells was analysed in a Zeiss Axioscope (filter 15). Left: Fluorescence microscopy; right: transmitted light microscopy.

Figure 7: Isolation of E. coli cells with esterase activity from a mixture with EstA-negative cells

For the determination of the enrichment by magnetic sorting of cells showing EstA-activity induced cells of each selection round were analysed as to their esterase activity. P-nitrophenylcaprylate served as hydrolysable standard substrate. Whereas the starting mixture did not exhibit esterase activity, after the first selection round already, a slightly increased substrate hydrolysis could be seen. After the second selection round already, it has nearly reached the level of a corresponding sample of induced pBBX+ transformants of E. coli JM109.

Example 1:

Production of E. coli cells presenting esterase on their cell surface

Esterase EstA from *Pseudomonas aeruginosa* was used as an example (Wilhelm et al., *J. Bacteriol.* 181 (1999), 6977-6986). EstA is an esterase from *Pseudomonas aeruginosa* consisting of an aminoterminal cell surface exposed enzyme domain and a membrane anchor domain which is located in the outer membrane and which mediates a translocation of the aminoterminal domain by the outer membrane. The nucleotide sequence and the amino acid sequence of EstA is shown in Fig. 4.
The coding sequence of EstA (Figure 4) was inserted into the vector PBBX+ (Wilhelm et al., loc. cit.) and was thus brought under the control of the *lac* promoter. After transformation of the E. coli strain JM109 (Stratagene), *E. coli* cells were

obtained which were able to hydrolyse the esterase substrate octanoic acid-p-nitrophenylester (Wilhelm et al., loc. cit.). The cell surface presentation of EstA was detected by immunofluorescent staining of the cells by successive incubation with anti-EstA antibodies (from rabbit), a biotinylated anti-rabbit antibody and streptavidin, phycoerythrin conjugate. Cells treated in that way showed red fluorescence during analysis in the fluorescence microscope (Zeiss Axioskop, filter 15) (Figure 5).

Example 2:

Coupling of Horseradish Peroxidase (HRP) on the surface of E. coli cells

10 µl sodium periodate solution (0.088 M) were added to 100 µl horseradish peroxidase (10 mg/ml). The solution was shaken at room temperature in the dark for 1 hour. Then, the enzyme was separated from the excess of sodium periodate by gel filtration via a Sephadex G25 column, diluted in 2 ml PBS buffer and stored at –20°C.

Example 3:

Synthesis of the EstA substrate LC-LC-biotin-tyramide octanoic acid ester

The EstA substrate (Figure 3) was produced from LC-LC-biotin-tyramide by reaction of 0.2 mg (1.17 µmol) octanoylchloride with 0.34 mg (0.57 µmol) LC-LC-biotin-tyramide (Pierce) into 0.2 ml pyridine. After 60 min incubation at room temperature, the reaction solution was lyophilised and resuspended in 0.1 ml dimethylformamide.

Example 4:

Esterase-mediated deposition of biotin on the surface of E. coli cells

At an optical density of 0.4, IPTG (end concentration 1 mM) was added to *E.coli* JM109 cells carrying the plasmid pBBX+ for the induction of the expression of the estA gene and incubated at 37°C for 60 min. *E. coli* JM109 cells not containing an estA gene were used as control. 500 µl of the cultures of both cell types were

centrifuged, coupled with oxidised peroxidase, subsequently washed with 500 µl PBS three times each and incubated in 500 µl 100 mM potassium phosphate buffer containing 0.0029 µmol biotin-LC-LC-tyramide-octanoic acid as well as 0.001% H_2O_2 for 15 min. The cells were centrifuged and re-suspended in 10 µl PBS containing 1 µl streptavidin, R-phycoerythrin conjugate (Molecular Probes). After incubation for 5 minutes, the cells were centrifuged and the supernatant was discarded. The cells were washed in 500 µl PBS buffer. Then, an aliquot was analysed in the fluorescence microscope (Zeiss Axioskop, filter 15). A deposition of biotin-tyramide was only detected in the cells with EstA activity (Figure 6).

Example 5:

Isolation of cells with esterase activity from a $1:10^6$ mixture with esterase-negative cells

To find out whether it is possible to isolate cells with esterase activity from a collection of cells without esterase activity, *E. coli* JM109 cells induced with IPTG containing the plasmid pBBR1MCS which does not comprise any *estA* gene, and JM109 cells containing the plasmid pBBX+ which contains an estA gene were mixed at a ratio of 10^6:1. Subsequently, the $1:10^6$ mixture was marked with the biotin-LC-LC-tyramide-octanoic acid ester as described above. Again 0.0029 µmol substrate in 500 µl potassium phosphate buffer were used. The incubation period was again 15 min. Then, the cells were centrifuged and washed with 500 µl PBS buffer three times. Then, 20 µl streptavidin-coated paramagnetic beads (Miltenyi Biotech, Bergisch Gladbach) were added to 200 µl of the cell suspension. After 15 min incubation, the cells were centrifuged and washed with 500 µl PBS three times, each. The cells were re-suspended in 500µl PBS. Subsequently, the suspension was passed through a column filled with iron beads and located within a strong magnetic field (MidiMacs, Miltenyi Biotech Bergisch Gladbach). The column was washed with 2 ml PBS buffer three times, each. Cells retained in the column were eluted with 2 ml PBS buffer after removal from the magnetic field. The cells were plated on selective plates and propagated over night. The following day,

the cells were detached and the process was repeated.

After each selection round, the sorted cells were harvested with 2 ml dYT each from the plates and grown again. For this purpose, 50 µl of harvested cells were inoculated in 50 ml dYT-Cm^{25}, each. After induction of the culture, the next marking and sorting round started. In this way, three selection rounds were carried out.

For the analysis of the enrichment, induced cells of each selection round were analysed as to their esterase activity. P-nitrophenylcaprylate served as hydrolysable standard substrate. Whereas the starting mixture did not exhibit esterase activity, after the first selection round already, a slightly increased substrate hydrolysis could be seen. After the second selection already, it has nearly reached the level of a corresponding sample of induced pBBX+ transformants of E. coli JM109.